MDR1 VARIANTS AND METHODS FOR THEIR USE

REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional applications no. 60/261,578 (filed January 12, 2001) and no. 60/314,829 (filed August 24, 2001), both of which are incorporated herein by reference in their entirety.

FIELD

This disclosure relates to methods and kits for detecting a subject's sensitivity to pharmaceutical agents, particularly an animal's sensitivity to application of drugs (such as ivermectin) that interact with P-glycoprotein. It also relates to variants of the *mdr1* gene, which variants impact transport of drugs that interact with the P-glycoprotein, as well as cell and whole animal systems comprising such variants and methods of using these systems.

BACKGROUND

The observation, over 100 years ago, that certain chemical dyes injected into the peripheral circulation were able to gain access to most organs but not the brain led to the concept of a blood-brain barrier. Research in the 1960's demonstrated that the anatomical basis of the blood-brain barrier is the specialized endothelial cells of brain capillaries. While it has been thought that the entry of drugs, toxins, and xenobiotics into the brain is simply a function of lipophilicity, electrical charge, and molecular weight, ongoing research demonstrates that the capillary endothelium composing the blood-brain barrier is not simply an anatomic entity. A number of active transport systems exist that selectively regulate both influx and efflux of compounds across brain capillary endothelial cells. The most important drug-efflux system of the blood-brain barrier identified to date is P-glycoprotein.

P-glycoprotein, the product of the *mdr1* (multidrug resistance) gene, is a 170-kD membrane-spanning, cell-surface protein that functions as a drug-efflux pump. P-glycoprotein was first identified over 20 years ago in chemotherapeutic drug-resistant tumor cells, and is now known to be a major cause of multidrug resistance in human and veterinary cancer patients. In tumor cells, P-glycoprotein functions as an ATP-dependent efflux pump resulting in decreased intracellular drug accumulation and reduced cytotoxicity. Chemotherapeutic drugs that are substrates for P-glycoprotein include *Vinca* alkaloids (vincristine and vinblastine), doxorubicin and related compounds, taxanes, and epipodophyllotoxins. Alkylating agents, platinum compounds, and antimetabolites are not substrates for P-glycoprotein. Though these agents are structurally and functionally dissimilar, P-glycoprotein substrates share several other characteristics. They typically are complex, hydrophobic, amphipathic compounds that are natural products (*i.e.*, derived from plants or micro-organisms) or analogs of natural products. A number of non-cytotoxic compounds have been identified as P-glycoprotein substrates, including steroid hormones, bilirubin, antiparasitic agents, selected antimicrobial agents, and others.

P-glycoprotein is expressed not only in tumor cells, but also in a variety of normal tissues, including renal tubular epithelium, canalicular surfaces of hepatocytes, adrenal cortical cells, colonic and intestinal epithelium, placenta, apical margins of bronchiolar epithelium, and brain capillary endothelial cells. Consistent with its function as a transport pump, the expression of P-glycoprotein in non-neoplastic tissues suggests a normal physiologic role for P-glycoprotein mediating the export of potentially toxic xenobiotics from the body. Although the normal function of P-glycoprotein in many of these tissues has not been elucidated, a great deal is known about its role in the blood-brain barrier.

Avermectins are a class of natural products with broad antiparasitic activity. Ivermectin, a semi-synthetic lactone in the avermectin family, is a drug that is used extensively in veterinary medicine to treat and control infections caused by nematode and arthropod parasites. It is also used in human medicine to treat onchocerciasis, lymphatic filariasis, and strongyloidiasis. Ivermectin induces a tonic paralysis in invertebrate organisms by potentiating glutamate-gated chloride channels, and/or gamma-amino butyric acid (GABA)-gated chloride channels (Tracy and Webster, In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th edition.* Hardman *et al.*, eds. New York: McGraw-Hill, 1996: 1009-1026, 1996) of the peripheral nervous system. In most mammals, the blood-brain barrier prevents access of ivermectin to the central nervous system, and since GABA receptors in mammals are restricted to sites within the central nervous system, mammals are generally protected from the neurologic effects of ivermectin (Fisher and Mrozik, *Annu. Rev. Pharmacol. Toxicol.* 32:537-553, 1992).

There are some specific subgroups of mice and dogs, however, that are exquisitely sensitive to the neurologic actions of ivermectin. Genetically engineered *mdr1a* knock-out [*mdr1a*(-/-)] mice are 50 to 100 times more sensitive to ivermectin-mediated neurotoxicity than wild-type mice (Schinkel *et al.*, *Cell.* 77:491-502, 1994), and accumulate 80-90-fold higher concentrations of ivermectin in the brain than do wild-type mice. The protein product of *mdr1a*, called P-glycoprotein (P-gp) is a 170-kD transmembrane protein pump that is present at high concentrations in the apical membrane of brain capillary endothelial cells (Van Asperen *et al.*, *J. Pharmaceut. Sci.* 86:881-884, 1997, 1997; Tsuji, *Therap. Drug Monitor.* 20:588-590, 1998). Substrates of P-gp include a variety of large, structurally unrelated hydrophobic compounds, including naturally occurring compounds such as ivermectin, cyclosporin, digoxin, and others. After substrates are bound by P-gp, they are actively extruded from the endothelial cell into the capillary lumen (Van Asperen *et al.*, *J. Pharmaceut. Sci.* 86:881-884, 1997). Abrogation of P-gp results in failure of the blood-brain barrier. High concentrations of ivermectin accumulate in brain tissue from mdr1a (-/-) mice, and neurotoxicity ensues.

Approximately 25% of a population of the CF-1 mouse strain were much more sensitive to neurotoxicity produced by ivermectin than unaffected mice of the same strain (Umbenhauer *et al.*, *Toxicol Appl. Pharmacol.* 146:88-94, 1997). Investigation into the cause of this sensitivity led to the discovery that the sensitive animals did not express P-gp in their brain endothelial cells.

Furthermore, a restriction-fragment-length polymorphism in the murine *mdr* gene was documented that allowed prediction of sensitive animals, and an inheritance pattern following normal Mendelian genetics was observed (Umbenhauer *et al.*, *Toxicol Appl. Pharmacol.* 146:88-94, 1997).

In dogs, a breed-related sensitivity to ivermectin has been reported in Collies, that may affect 30 to 50% of the population (Pulliam *et al.*, *Veter. Med.* 7:33-40, 1985; Hsu *et al.*, *Comp. Contin. Educat. Veter. Pract.* 11:584-589, 1989, Paul *et al.*, *Am. J. Vet. Res.* 48:685-688, 1987). In one study, 1/200th of the lethal dose of ivermectin for Beagles was lethal for Collies (Pulliam *et al.*, *Veter. Med.* 7:33-40, 1985). Other related canine breeds believed to be affected by ivermectin sensitivity include Border Collies, Shetland Sheepdogs, Old English Sheepdogs, and Australian Shepherds (Campbell and Benz, *J. Vet. Pharmacol. Therap.* 7:1-16, 1984).

Despite numerous investigations (Vaughn, *et al.*, *Vet. Res. Commun.* 13:47-55, 1989; Roher *et al.*, *Vet. Res. Commun.* 14:157-165, 1990; Pulliam *et al.*, *Veter. Med.* 7:33-40, 1985), the mechanism for ivermectin-sensitivity in Collies is unknown.

SUMMARY OF THE DISCLOSURE

The disclosure provides a mutation in the *mdr1* gene, which results in production of truncated and non-functional P-gp and thereby causes sensitivity to ivermectin and other drugs that serve as P-gp substrates. With the identification of this mutation, methods are provided to determine if an individual subject is sensitive to ivermectin. Also provided are systems for examining the importance of P-gp in drug transport in whole animal and cell culture systems.

A provided embodiment is a method of detecting ivermectin sensitivity in a subject (for instance a mammal, such as a canine animal), which method includes determining whether a gene-truncation mutation in a *mdr1*-encoding sequence of the subject or a truncated P-pg is present in the subject. Such a gene-truncation mutation or truncation of P-gp indicates that the subject is sensitive to ivermectin. In specific examples of such methods, the gene truncation mutation is a deletion of about four base pairs at about residue 294-297 of SEQ ID NO: 1 (the canine mdr1 cDNA) or a homologous cDNA or gene.

In certain embodiments, methods provided herein are used to evaluate whether the subject can be treated safely with ivermectin or another drug that can be excluded from the brain by P-gp (such as those listed in Table 2).

In certain provided methods, the method includes determining whether the subject is homozygous or heterozygous for the gene-truncation mutation.

In specific examples of the provided methods, determining whether a gene-truncation mutation is present in the subject includes subjecting DNA or RNA from the subject to amplification using oligonucleotide primers, for instance in performing an oligonucleotide ligation assay.

In a specific embodiment provided herein, the method of detecting ivermectin sensitivity in a subject involves obtaining a test sample of DNA containing a *mdr1* sequence of the subject; and determining whether the subject has the gene-truncation mutation in the *mdr1* sequence, wherein the

presence of the mutation indicates sensitivity to ivermectin. In certain examples of this embodiment, determining whether the subject has the mutation comprises using restriction digestion, probe hybridization, nucleic acid amplification, or nucleotide sequencing.

Further embodiments of methods provided herein involve obtaining from the subject a test sample of DNA comprising an *mdr1* sequence; contacting the test sample with at least one nucleic acid probe for an *mdr1* gene truncation mutation that is associated with ivermectin sensitivity, to form a hybridization sample; maintaining the hybridization sample under conditions sufficient for specific hybridization of the *mdr1* sequence with the nucleic acid probe; and detecting whether the *mdr1* sequence specifically hybridizes with the nucleic acid probe, wherein specific hybridization of the *mdr1* sequence with the nucleic acid probe indicates ivermectin sensitivity. In specific examples of such embodiments, the probe is present on a substrate, for instance a nucleotide array.

Also provided are methods of detecting ivermectin sensitivity in a subject by determining whether truncated P-gp is present in a sample from the subject. Certain examples of such methods will involve reacting at least one P-gp molecule contained in the sample from the subject with a P-gp-specific binding agent (such as an antibody) to form a P-gp:agent complex. Such methods can further include detecting the P-gp:agent complex, for instance by Western blot assay, ELISA, or other immunoassay technique.

Also provided herein are kits for use in diagnosing ivermectin sensitivity in a subject. Such kits include at least one probe that specifically hybridizes to an *mdr1* gene-truncation mutation associated with ivermectin sensitivity. In specific examples of such kits, the probe specifically hybridizes to an *mdr1* gene-truncation mutation at or about residue 294-297 of SEQ ID NO: 1.

Other provided kits for use in diagnosing ivermectin sensitivity in a subject contain a P-gp-specific binding agent, such as an antibody. In specific examples of such kits, the provided agent is capable of specifically binding to truncated P-gp protein.

Also provided herein are oligonucleotides that specifically hybridize to a canine *mdr1* gene-truncation mutation, for instance an oligonucleotide that hybridizes to an *mdr1* gene-truncation mutation at residue 294-297.

Other embodiments are systems and methods for studying the effects of drugs (and drug candidates) on biological systems expressing a *mdr1* gene truncation, such as the *mdr1* gene-truncation mutation at residues 294-297. Examples of such systems include cultured cells (such as intestinal epithelial cells, brain endothelial cells (for instance, capillary endothelial cells), renal-tubular cells, hepatocytes, or neoplastic cells) isolated from a canine that naturally exhibits a gene truncation mutation in the *mdr1* gene. Other examples of such systems include animal models (including for instance dogs) in which the *mdr1* gene is naturally truncated, or in which such truncations have been engineered using recombinant technologies and/or cloning. Methods are also provided for using these animal models and cell systems, for instance to study drug interactions with P-gp or to examine the impact of drugs and drug candidates on biological systems. Such methods would be particularly useful in the drug approval process.

One embodiment is a method of determining a P-gp influenced biological effect of a compound on a canine cellular system, which method involves contacting a canine cell having a truncation mutation in its *mdr1* gene with the compound, and comparing a characteristic (such as a genetic, physiological, chemical, or morphological characteristic) of the canine cell contacted with the compound with the characteristic of a similar canine cell that was not contacted with the compound. In such methods, a difference in the characteristic between the two cells is indicative of the P-gp influenced biological effect in the cell. In specific examples of such methods, the canine cell is a Collie cell. The truncation mutation in the *mdr1* gene is in some examples a mutation at residue 294-297.

Specific types of canine cells include, but are not limited to, gastrointestinal tissue cells, renal tissue cells, nerve tissue cells, brain capillary endothelial cells, and liver tissue cells. In some methods, the canine cell is a neoplastic cell.

Also provided are methods of determining a P-gp influenced biological effect of a compound on a canine cellular system, wherein contacting the canine cell with the compound occurs *in vivo* in the native environment of the canine cell, for instance in a dog (such as a Collie dog).

In some examples of the provided methods, biological effects include absorption or distribution of a drug or compound, for instance a drug or compound that interacts with or is transported by P-gp.

Also provided is an animal model useful for studying a P-gp influenced biological effect of a compound, comprising a Collie identified as being homozygous or heterozygous for a truncation mutation in the *mdr1* gene (for instance, a mutation at residue 294-297). Also provided are methods of using this animal model to examine the effect of compounds that interact with P-gp, for instance compounds that are transported by P-gp or that modulate its transport activity.

Compounds contemplated for use in the methods provided herein include anti-infective agents (*e.g.*, antiviral, antibacterial, or anti-prion agents), antineoplastic agents, analgesics, neurokinin receptor antagonists, anti-emetic agents, beta-adrenergic receptor antagonists, antiepileptic agents, anti-psychotic agents, anti-depressive agents, and other drugs that act on the central nervous system.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1A is a semi-quantitative reverse transcriptase PCR analysis of *mdr1* in blood samples from ivermectin-sensitive (samples 15 and 16) and non-sensitive (resistant) (samples 17 and 18) Collies. Duplicate reactions were assayed for each dog as shown using canine *mdr1* and GAPDH primers. The resultant amplicons were separated by electrophoresis through an agarose gel. The arrows indicate the expected size of the mdr1 (P-gp) and GAPDH products.

FIG. 1B is a graph, quantitating the UV fluorescence of *mdr1* and GAPDH reverse transcriptase PCR products obtained by digital image analysis of the ethidium bromide-stained gel shown in FIG 1A.

FIG. 2 is a sequence comparison of wild-type (top) and mutant (bottom) *mdr1* cDNAs. As demonstrated herein, a four base pair deletion is present in the mutant cDNA. Codons in the vicinity of the deletion are indicated by brackets for both the wildtype and mutant cDNAs. Bolded letters indicate stop codons created in the mutant cDNA as a result of the frame-shift. The dashed box indicates the palindromic sequence in the vicinity of the deletion mutation.

FIG. 3 is a diagrammatic representation of the transmembrane structure of P-gp (Gottesman and Pastan, *Annu. Rev. Biochem.* 62:385-427, 1993). The mutation site (arrowhead) occurs at amino acid 75, resulting in a frame shift that generates several downstream stop codons, the first two of which occur at amino acid positions 91 and 111. Greater than 90% of the protein is predicted to be missing in dogs homozygous for the mutant allele due to the truncation.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three-letter code for amino acids, as defined in 37 C.F.R. §1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. In the accompanying sequence listing:

SEQ ID NO: 1 shows the nucleotide sequence of the canine *mdr1* CDNA (GenBank Accession No. AF045016), and the P-gp amino acid sequence encoded thereby.

SEQ ID NO: 2 shows the amino acid sequence of canine P-gp.

SEQ ID NOs: 3-10 show respective synthetic oligonucleotides used to primer *in vitro* amplification reactions of the canine *mdr1* gene, as described in Example 1.

DETAILED DESCRIPTION

I. Abbreviations

mdr	multidrug resistance gene
P-gp	P-glycoprotein, protein product of the *mdr1* gene

II. Terms

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes VII*, published by Oxford University Press, 2000 (ISBN 0-19-879276-X); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

Unless otherwise explained herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention

belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

III. Identification of truncation mutations in mdr1, responsible for Collie sensitivity to ivermectin.

There is a sub-population of Collies and dogs of related breeds that display a similar sensitivity to ivermectin. The inventors have surprisingly discovered that a deletion mutation of the *mdr1* gene exists in ivermectin-sensitive Collies. The mutation produces a frame shift that generates a premature stop codon in the *mdr1* gene resulting in a severely truncated, nonfunctional protein. Collies homozygous for the deletion (mutant/mutant) exhibit ivermectin sensitivity while those that are heterozygous (normal/mutant) or homozygous normal are not sensitive to ivermectin neurotoxicity. Several other breeds, including Australian shepherds, Shelties, and Old English Sheepdogs have been reported to exhibit ivermectin sensitivity; it is believed that susceptible individuals of these species may also display truncation mutations in the *mdr1* gene.

The identification of the naturally occurring *mdr1* truncation mutations in dogs has enabled the use of animals carrying such a mutation, and of cells derived from such animals, to study interactions of drugs (or potential drugs) with P-gp and the systemic effects of such interactions. These animal and cell-based systems are particularly useful for identifying and characterizing ways to:

(a) improve, regulate, or prevent gastrointestinal absorption of drugs;

(b) improve, regulate, or prevent brain penetration of drugs (*e.g.*, increasing brain penetration of HIV-1 drugs; drugs for treating prion diseases; antineoplastic agents; or compounds for pain or depression);

(c) improve, regulate, or prevent renal excretion of drugs;

(d) improve penetration of drugs into tumor cells;

(e) improve, regulate, or prevent biliary excretion of drugs; and

(f) improve, regulate or prevent penetration of drugs through the placenta.

These systems are also particularly useful to study the effects of functional *mdr1* polymorphism(s), particularly in order to understand the effects of such polymorphisms as they may be found in additional species, including for instance humans. The systems can also be used to study the effects (drug interactions) that a highly effective, potent P-gp inhibitor would have in an intact biological

system, and thus provides an excellent method for examining the large-mammal effects of drugs, for example during the drug approval and testing process.

The invention is illustrated by the following non-limiting Examples.

EXAMPLES

Example 1: Identification of a Truncation Mutation in Collie mdr1

Materials and Methods

General Procedures and Materials

Procedures used in research disclosed herein were approved by the Institutional Animal Care and Use Committee.

Blood samples (8 ml) were obtained by jugular venipuncture from each of 13 clinically healthy Collies (Wil-O-Lane Kennels, Allegan, MI). All Collies used in the study had previously been identified as ivermectin-sensitive or -nonsensitive by observing dogs for signs of neurotoxicosis after oral administration of 120 µg of ivermectin per kg of body weight (20 times the label dose for heartworm prevention), as previously described (Paul *et al.*, *Amer. J. Vet. Res.* 61:482-483, 2000; Fassler et al., *J. Am. Vet. Med. Assoc.* 199:457-460, 1991). Each dog was identified by an ear tattoo and housed in a run measuring 4 x 8 feet with metal walls and a raised, coated, metal screen floor. Facilities exceeded the minimal requirement specified by USDA guidelines.

Seven Collies were identified as ivermectin-sensitive (4 male and 3 female) and 6 were not ivermectin-sensitive (3 male and 3 female). Two of the Collies in the study were littermates and two others were non-littermate siblings. Of the two littermates, one was sensitive to ivermectin, the other was not. Both of the non-littermate siblings were sensitive to ivermectin. Several other Collies in this study shared either the same sire or the same dam. Two of the common sires and three of the common dams were represented by offspring in both the ivermectin-sensitive and -nonsensitive groups. Additional blood samples were obtained from 4 non-Collie dogs, including 1 Beagle, 2 Golden Retrievers, and 1 Staffordshire terrier cross-bred dog.

Semiquantitative RT-PCR of canine mdr1 gene

Total RNA was extracted from venous blood leukocytes using TRIzol reagent (Gibco BRL). Blood leukocytes were prepared by density gradient centrifugation. For RT reactions, a GeneAmp RT PCR kit (Perkin-Elmer) was used with oligo(dT) primers. Equivalent amounts of cDNA were then amplified in separate PCR reactions using Amplitaq (Perkin Elmer) with 2.5 mM $MgCl_2$.

PCR consisted of either 20 (GAPDH) or 27 (*mdr1* product A) cycles, with denaturing, annealing, and extension conditions of 95° C (15 seconds), 60° C (15 seconds), and 72° C (60 seconds) in a Perkin-Elmer thermocycler (2400 GeneAmp PCR System). The number of cycles for each product was determined on the basis of kinetic studies to ensure that the amplification reaction

was within the logarithmic (not plateau) range. PCR products were resolved by electrophoresis in 1% agarose gels containing ethidium bromide. Expected sizes of the GAPDH and *mdr1* A band are 229 bp and 892 bp, respectively. The UV fluorescence of DNA bands was measured with an IS-1000 Digital Imaging System (Alpha Innotec). For individual dogs, the fluorescence value for the *mdr1* A product is divided by the fluorescence value of the GAPDH product to allow direct comparison between dogs.

Sequencing of canine mdr1 cDNA

Four primer pairs, amplifying four products (referred to herein as A, B, C, and D) spanning 95% of the canine *mdr1* cDNA (GenBank Accession No. AF04016) were designed for sequencing experiments (see, Table 1). Using RNA from three of the sensitive Collies, cDNA was synthesized in RT reactions as described above and amplified in separate reactions. For products A, B, and C, PCR was accomplished using the conditions described above, using 35 cycles. PCR for product D consisted of 35 cycles, with denaturing, annealing, and extension conditions of 95° C (10 seconds), 64.5° C (15 seconds), and 72° C (150 seconds). Optimal $MgCl_2$ concentrations were 2.5 mM for PCR products A, C, and D, and 1.5 mM for PCR product B. For initial experiments, products generated from samples of three different dogs were ligated into pGEM-T Easy (ProMega), which was then used to transform ElectroMAX DH10B *E. coli* cells by electroporation (Gene Pulser II, BioRad). Plasmid DNA was isolated (Plasmid Mini Kit, Qiagen) and sequenced by Davis Sequencing Inc. (Davis, CA) using dye-terminator chemistry and an automated DNA sequencer (ABI 377, PE Applied Biosystems). For all subsequent experiments, sequencing of PCR products (Davis Sequencing Inc.) following purification (Qiaquick PCR Purification Kit, Qiagen) was performed. Sequences from experimental dogs were compared to the known canine *mdr1* sequence (GenBank AF 045016; SEQ ID NO: 1).

Table 1. Oligonucleotide primers used in this study. The combination of primer pairs used in this study provides >95% coverage of the canine *mdr1* cDNA.

***Mdr1* product designation**	**Primer**	**Position**	**Size of PCR Product**
A	Forward: 5'- TCC GGT TTG GTG CCT ACT TG[1]	2942-2961	892
	Reverse: 5'- TGC TCC TTG ACT TTG CCA TTC[2]	3833-3814	
B	Forward: 5'- CCT CAC TAA GCG GCT TCG ATA C[3]	2421-2441	1021
	Reverse: 5'- AAA CAG GAT GGG CTC CTG AGA C[4]	3441-3420	
C	Forward: 5'- CAG CAC GTT TGC AAT GTT TC[5]	168-189	1061
	Reverse: 5'- TCT GGT TTA TGT CCA CTC TTC G[6]	1228-1208	
D	Forward: 5'- AGG CAT CCC CAA GCA TTG AAG[7]	1112-1132	1432
	Reverse: 5'- TGA GCC GCA TCA TTG GCA AG[8]	2543-2524	

[1] Corresponds to SEQ ID NO: 3.
[2] Corresponds to SEQ ID NO: 4.
[3] Corresponds to SEQ ID NO: 5.
[4] Corresponds to SEQ ID NO: 6.
[5] Corresponds to SEQ ID NO: 7.
[6] Corresponds to SEQ ID NO: 8.
[7] Corresponds to SEQ ID NO: 9.
[8] Corresponds to SEQ ID NO: 10.

Results

We took advantage of a well-defined population of ivermectin-sensitive and non-sensitive Collies (Paul *et al.*, *Amer. J. Vet. Res.* 61:482-483, 2000: Fassler *et al.*, *J. Am. Vet. Med. Assoc.* 199:457-460, 1991). Sensitive animals were designated as those that experienced clinical signs of neurologic toxicity after receiving a single, oral dose of ivermectin (120 µg/kg). Clinical signs of neurotoxicity that were evaluated include apparent depression, ataxia, mydriasis, salivation, or drooling (Paul *et al.*, *Amer. J. Vet. Res.* 61:482-483, 2000). Semi-quantitative reverse transcriptase PCR analysis was conducted on RNA isolated from ivermectin-sensitive and non-sensitive Collies to determine if *mdr1* expression is lower in sensitive Collies than in non-sensitive Collies. Amplification of a 229-bp product of the canine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an internal standard to control for variability in reverse transcriptase and PCR reactions. FIG. 1A shows representative ethidium bromide-stained RT-PCR products of GAPDH (229 bp) and *mdr1* (product A, 892 bp) from ivermectin sensitive (samples 15 and 16) and nonsensitive (samples 17 and 18) Collies. Individual UV fluorescence intensity values for GAPDH and *mdr1* cDNA derived from these gels are shown in FIG. 1B. The level of *mdr1* gene expression, as determined by semiquantitative reverse transcriptase PCR, did not differ between ivermectin-sensitive and non-sensitive Collies.

A reverse transcriptase PCR strategy was used to clone *mdr1* cDNA from one normal dog (beagle) and 3 ivermectin-sensitive Collies. Sequence data from the beagle *mdr1* cDNA was identical to that reported for normal canine *mdr1* cDNA (GenBank Accession No. AF045016). Sequence analysis of clones from the three ivermectin-sensitive Collies revealed an identical four base pair deletion within the first 10% of the transcript (FIG. 2). This deletion causes a frame-shift mutation at nucleotide 294 of the transcript, which corresponds to amino acid 75 (FIG. 3). The frame shift generates several stop codons (FIG. 3), the first of which occurs at amino acid position 91 of P-gp (GenBank Accession No. AF04016; SEQ ID NO: 2). The result is a severely truncated, nonfunctional, protein.

For all subsequent experiments, a PCR-based strategy was used for sequencing *mdr1* cDNA so that heterozygosity could be readily detected. The exact four-nucleotide deletion (AGAT) was detected in all (7/7) samples from ivermectin-sensitive Collies. Furthermore, these dogs were homozygous for the deletion. Samples from all non-Collie dogs (1 Beagle, 2 Golden Retrievers, and 1 Staffordshire terrier cross-bred dog) were homozygous wild-type. Interestingly, samples from all (6/6) ivermectin non-sensitive Collies displayed a heterozygous genotype, with one strand carrying the mutant allele, and the other strand carrying the wild-type allele. It is highly unlikely that these findings are a result of chance (Fisher's Exact test, $P = 0.006$).

Discussion

The reported data demonstrate that a frame-shift deletion of four base pairs at the 5' end of the canine *mdr1* gene is associated with (and likely causes) ivermectin-sensitivity in Collies.

Premature termination of P-gp synthesis as a result of the frame-shift yields a severely truncated protein that is less than one-tenth its normal size, based on the predicted amino acid sequence. P-glycoprotein's drug-efflux function is dependent upon ATP binding sites, substrate binding sites, phosphorylation sites, and multiple membrane-spanning motifs (Yoshimura *et al.*, *J. Biol. Chem.* 264:16282-16291, 1989; Skach and Lingappa, *Cancer Res.* 54:3202-3209, 1994). Since none of these required elements are present in the truncated protein, we conclude that animals homozygous for the deletion do not express a functional form of P-gp.

The pivotal role that P-gp plays in protecting the brain from ivermectin has previously been established (Kwei *et al.*, *Drug Metab. Dispos.* 27:581-587, 1999; Marques-Santos *et al.*, *Pharmacol. Toxicol.* 84:125-129, 1999; Schinkel *et al.*, *J. Clin. Invest.* 97:2517-2524, 1996; Van Asperen *et al.*, *J. Pharmaceut. Sci.* 86:881-884, 1997). Because a large number of other drugs serve as P-gp substrates, it is possible that affected Collies would also experience greater sensitivity to the neurologic effects induced by drugs other than ivermectin. Examples of P-gp substrates that might induce neurotoxicity include those listed in Table 2 (Tsuji, *Therap. Drug Monitor.* 20:588-590, 1998; Schinkel *et al.*, *J. Clin. Invest.* 97:2517-2524, 1996).

Table 2. Selected Clinically Relevant Substrates of P-glycoprotein

Antineoplastic agents	**Antimicrobials and Antiparasitics**	**Miscellaneous**
Vincristine*	Cefoperazone	Digoxin*
Vinblastine*	Tetracylines	Cyclosporine A
Doxorubicin*	Ivermectin*	Verapamil*
Mitoxantrone		Loperamide*
Paclitaxel*		Dexamethasone
		Ondansetron*

*Drugs that have potential for neurotoxicity

In mice, people, and dogs, P-gp is normally expressed in other tissues of the body in addition to brain capillaries. Consistent with its function as a transport pump, expression of P-gp occurs at sites where it might protect the animal from xenobiotics. For example, P-gp is expressed at high levels in renal proximal tubular cells, liver, small bowel, colon, and placenta (as well as brain endothelium) (Lum *et al.*, *Pharmacother.* 13:88-109, 1993; Ginn, *Vet. Pathol.* 33:533-541, 1993). The highest levels of P-gp expression occur in tumor cells, where P-gp functions as a multidrug transporter, protecting tumor cells from a variety of chemotherapeutic drugs including anthracyclines, *Vinca* alkaloids, taxanes, and epipodophyllotoxins. It is believed that ivermectin-sensitive Collies would be less likely to develop multidrug resistant tumors than the general canine population, and would therefore have better chemotherapy response rates.

The specific cause of the identified mdr1 mutation is unknown. However, it has been reported that unusual DNA structures, including palindromic DNA, promote genetic instability (Lewis *et al.*, *Ann. NY Acad. Sci.* 870:45-57, 1999). Unusual DNA structures are thought to cause DNA polymerase to pause and, consequently, can disrupt DNA replication. Mutational events are

not limited to sequences located within the palindromic DNA, but can also occur in sequences in the vicinity of a palindrome. Therefore, it is interesting to note that a palindromic sequence (GGTTTTTGG; FIG. 2) occurs nine bases upstream of the deletion site. Whether or not this palindromic sequence played a role in generating the four-base pair deletion in these Collies is unknown.

The inheritance pattern of ivermectin sensitivity in Collies is unknown. Results of the research described here are consistent with an autosomal recessive inheritance pattern, since only Collies that had two mutant alleles displayed the ivermectin-sensitive phenotype. However, a larger sample size will be needed to definitively determine the inheritance pattern. With many genetic diseases in people, affected individuals have many different mutations throughout the affected gene. However, in this study, all Collies had the same mutation. Because many of these animals were unrelated, it seems likely that the mutant alleles in these dogs have a common, yet-to-be-determined origin.

Example 2: Frequency of the mutant MDR1 allele

This Example provides methods and results from a study to determine the frequency of the mutant MDR1 allele associated with ivermectin sensitivity in a sample of Collies living in Washington and Idaho. Blood was collected from 40 Collies for RNA extraction. The RNA was reverse transcribed, and PCR performed to amplify a 1061-base pair amplicon containing the MDR1 mutation. Sequence analysis was performed to determine the genotype of each dog. Twenty-two percent of the Collies studied were homozygous for the normal allele (normal), 42% were heterozygous (carrier), and 35% were homozygous for the mutant allele (affected).

Materials and Methods

Animals -- Forty clinically healthy, client-owned Collies were studied. Owner consent was obtained, and the study was approved by the Institutional Animal Care and Use Committee. Both rough-coated and smooth-coated Collies were represented. Dogs included in the sample population were those animals for which the owner was interested in determining MDR1 genotype in their pet(s). Advertising for Collies occurred primarily by announcements at the Inland Northwest Collie Club meetings and word-of-mouth. A pedigree (representing the last 4 generations) was available for eight animals.

Collection and Extraction of RNA -- An 8 ml blood sample was collected from each dog for RNA isolation. Blood leukocytes were prepared by density gradient centrifugation. Total RNA was extracted from venous blood leukocytes using TRIzol reagent (Gibco BRL).

Reverse transcriptase PCR and sequencing -- For reverse transcriptase reactions, a GeneAmp RT PCR kit (Perkin-Elmer) was used with oligo(dT) primers. The cDNA was then amplified (using primers as described in Example 1) in separate PCR reactions using Amplitaq (Perkin Elmer) with 2.5 mM $MgCl_2$. PCR consisted of 36 cycles, with denaturing, annealing, and

extension conditions of 95° C (10 seconds), 64° C (15 seconds), and 72° C (60 seconds) in a MJ Research thermocycler (PTC-200). PCR products were resolved by electrophoresis in 1% agarose gels containing ethidium bromide. Expected size of the MDR1 band was 1061 bp. PCR products were purified (Qiaquick PCR Purification Kit, Qiagen) and sequenced by Davis Sequencing Inc. (Davis, CA) using dye-terminator chemistry and an automated DNA sequencer (ABI 377, PE Applied Biosystems). Sequences from experimental dogs were compared to the known canine MDR1 sequence (GenBank AF 045016).

Results

The deletion mutation associated with ivermectin sensitivity in Collies was present in a large number of dogs in this study. Nine dogs (22%) were homozygous for the normal (wild-type) MDR1 sequence, 14 dogs (35%) were homozygous for the mutant allele, and 17 (42%) were heterozygous.

Analysis of the 8 pedigrees was performed and showed that four of the dogs in the study were closely related. For one pair of siblings, test results indicated that one dog was affected (homozygous mutant) and the other was heterozygous (one normal allele and one mutant allele). For another closely related pair of dogs, a dam and her daughter, test results indicated that the dam was heterozygous, and the daughter one dog was affected. However, there were two affected dogs that were not related to other dogs in the study within the four most recent generations. Furthermore, these eight dogs were unrelated (within the four most recent generations) to a sample population of Collies from Michigan that were studied previously, in which all animals were either heterozygous or homozygous for the described MDR1 mutation.

Discussion

Ivermectin sensitivity in Collies has recently been associated with homozygous expression of a deletion mutation of the MDR1 gene. P-glycoprotein, the product of the MDR1 gene, is an integral component of the blood-brain barrier. At the blood brain barrier, P-glycoprotein actively extrudes drugs from brain tissue back into capillaries, resulting in lower brain concentrations of drugs that are substrates for P-glycoprotein (Fromm,. *Int J Clin Pharm Therap* 38:69-74, 2000; Kim *et al.*, *J Clin Invest* 101:289-294, 1998; Jonker *et al.*, *Br J Pharmacol* 127:43-50, 1999; Schinkel, *Int J Clin Pharmacol Ther* 36:9-13, 1998). In MDR1 knockout mice, lack of P-glycoprotein leads to abnormally increased accumulation of certain drugs in the brain with resultant undesired neurologic adverse effects (Schinkel, *Int J Clin Pharmacol Ther* 36:9-13, 1998). In ivermectin-sensitive Collies, this mutation consists of a 4-base-pair deletion that generates a premature stop codon, resulting in a severely truncated, nonfunctional protein product.

Previous investigators have estimated that up to 30-40% of Collies are sensitive to ivermectin (Pulliam *et al.*, *Vet Med* 80:33-40, 1985; Paul *et al.*, *Am J Vet Res* 48:684-685, 1987; Rohrer and Evans, *Vet Res Commun* 14:156-165, 1990). Our study yielded similar results. In our study population, the frequency of the homozygous mutant genotype was 35%. Interestingly, in a

separate sample of Collies from Michigan, all dogs carried at least one mutant allele. From the pedigrees available, 8/40 from this sample and 15/15 from the Michigan sample, none of the Washington/Idaho dogs were related to the Michigan dogs within the four most recent generations. Collectively, these results suggest that the MDR1 mutation associated with ivermectin sensitivity is widely dispersed in the Collie population.

Sporadic descriptions of ivermectin sensitivity have been reported in a few other breeds including Shetland sheepdogs, Australian shepherds, and Old English sheepdogs (Hadrick *et al.*, *JAVMA* 206:1147-1150, 1995; Paradis, *Compend Cont Ed Pract Vet* 20:193-200, 1998; Hsu *et al.*, *Compend Cont Ed Pract Vet* 11:584-588, 1989). Whether or not these breeds share the same MDR1 mutation as Collies is unknown. In people, several different MDR1 mutations have been described, so it is reasonable to assume that other breeds may not share the same MDR1 genotype as do Collies (Cascorbi *et al.*, *Clin Pharmacol Ther* 69:169-174, 2001; Kerb *et al.*, *Pharmacogenomics* 1:51-64, 2001).

Determination of the genotype of Collies is important clinically for several reasons. First, ivermectin is not the only clinically relevant substrate for P-gp that can cause neurotoxicity. The over-the-counter antidiarrheal agent loperamide has been reported to cause neurotoxicity in Collies at doses routinely used in other breeds (Hugnet *et al.*, *Vet Hum Toxicol* 38:31-33, 1996). Loperamide, like ivermectin, is generally excluded from entering brain tissue in high concentrations by the actions of P-gp. In affected Collies, loperamide achieves high concentrations in brain tissue causing neurologic toxicity. In support of this fact, one of the Collies in the present study was treated with an appropriate dose of loperamide as a puppy and developed severe (nearly fatal) neurologic toxicity. The dog tested homozygous for the mutant allele. Other drugs that are substrates for P-gp and that can cause neurotoxicity in affected Collies include vincristine, vinblastine, ondansetron, and potentially moxidectin.

There are other, non-neurologic, implications for Collies with the MDR1 mutation described. P-glycoprotein is normally also expressed at the luminal border of the intestinal tract (Liu and Hu, *Clin Chem Lab Med* 38:877-881, 2000), where it functions as an "anti-absorption" mechanism for a number of drugs, including digoxin, cyclosporin A, dexamethasone, antiviral drugs and others (Wacher *et al.*, *Advanced Drug Delivery Rev* 46:89-102, 2001). In affected Collies, oral bioavailability of these drugs is likely to be greater than in unaffected dogs. This would result in higher plasma concentrations and a higher likelihood of adverse drug reactions in affected Collies.

It is likely that a high percentage of Collies presented to veterinarians for treatment are affected by the MDR1 mutation described in this report. It is important that veterinarians consider this factor when selecting pharmacological therapy for Collies. Furthermore, an adverse drug reaction involving neurologic toxicity should be considered for Collies exhibiting abnormal CNS signs.

Example 3: Other *mdr1* Truncations

With the provision herein of the correlation between a canine *mdr1* gene truncation and ivermectin sensitivity, the isolation and identification of additional *mdr1* truncations, and similar truncations in other canine species, is enabled. Conventional methods for the identification of genetic polymorphisms in a population can be used to identify such additional polymorphisms.

For instance, existing populations (*e.g.*, Collie or other populations) are assessed for ivermectin sensitivity (or sensitivity to other drugs that interact with P-gp), and a subset of individuals within the population (such as those subjects known to be prone to neurotoxicosis, or related individuals) are genotyped as relates to an *mdr1* sequence. These *mdr1* sequences are then compared to a reference *mdr1* sequence, such as the frame-shift truncation allele described herein, to determine the presence of one or more variant nucleotide positions. After variant nucleotides are identified, statistical analysis of the population can be used to determine whether these variants are correlated with sensitivity to ivermectin or other drug treatment.

Alternatively, the P-gp protein itself can be analyzed in such subjects, to determine the presence and/or level and/or size of the protein.

Example 4: Clinical Uses of *mdr1* Polymorphisms

To perform a diagnostic test for the presence or absence of a truncation mutation (*e.g.*, a deletion, frameshift, point mutation, or other change that results in a truncated protein product) in an *mdr1* sequence of an individual, a suitable genomic DNA-containing sample from a subject is obtained and the DNA extracted using conventional techniques. Most typically, a blood sample, a buccal swab, a hair follicle preparation, or a nasal aspirate is used as a source of cells to provide the DNA sample. The extracted DNA is then subjected to amplification, for example according to standard procedures. The allele of the truncation mutation is determined by conventional methods including manual and automated fluorescent DNA sequencing, primer extension methods (Nikiforov, *et al.*, *Nucl. Acids Res.* 22:4167-4175, 1994), oligonucleotide ligation assay (OLA) (Nickerson *et al.*, *Proc. Natl. Acad. Sci. USA* 87:8923-8927, 1990), allele-specific PCR methods (Rust *et al.*, *Nucl. Acids Res.* 6:3623-3629, 1993), RNase mismatch cleavage, single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), Taq-Man, oligonucleotide hybridization, and the like. Also, see the following U.S. Patents for descriptions of methods or applications of polymorphism analysis to disease prediction and/or diagnosis: 4,666,828; 4,801,531; 5,110,920; 5,268,267; and 5,387,506.

The markers of ivermectin sensitivity disclosed herein can be utilized for the detection of, and differentiation of, individuals who are homozygous and heterozygous for the truncation mutation polymorphism(s). The value of identifying individuals who carry a sensitive allele of *mdr1* (*i.e.*, individuals who are heterozygous or homozygous for an allele that contains a "sensitive" *mdr1* polymorphism, such as the truncation described herein) is that therapy for these individuals can then be initiated or customized (*e.g.*, through avoiding ivermectin or other drugs usually kept from

crossing the blood-brain barrier by P-gp) to reduce the occurrence of neurotoxicity in these individuals. Likewise, the presence of the allele will assist breeders in breeding programs, either to avoid introducing a sensitive allele into a breeding population, or by selectively avoiding animals carrying such an allele. Information regarding an animal's *mdr1* allele status (sensitive, resistant, or heterozygous) could for instance be tested early in the life of the individual, and included on a license, medical record, pedigree, and so forth.

Example 5: Polymorphism Gene Probes and Markers

Sequences surrounding and overlapping truncation polymorphisms in the *mdr1* gene can be useful for a number of gene mapping, targeting, and detection procedures. For example, genetic probes can be readily prepared for hybridization and detection of the described truncation polymorphism. Such probe sequences may be greater than about 12 or more oligonucleotides in length and possess sufficient complementarity to distinguish between a wild-type sequence and a sequence in which four nucleotides (*e.g.*, nucleotides corresponding to positions 294-297 of SEQ ID NO: 1) have been lost.

Similarly, sequences surrounding and overlapping the specifically disclosed truncation polymorphism (or other polymorphisms found in accordance with the present teachings) can be utilized in allele-specific hybridization procedures. A similar approach can be adopted to detect other *mdr1* polymorphisms, such as truncations.

Sequence surrounding and overlapping a *mdr1* polymorphism, or any portion or subset thereof that allows one to identify the polymorphism, are highly useful. Thus, another embodiment provides a genetic marker predictive of the herein-disclosed frame-shift truncation polymorphism of *mdr1*, comprising a partial sequence of the canine genome including at least about 10 contiguous nucleotide residues including residues 294-297 of SEQ ID NO: 1, or specifically not including these four residues but still including the surrounding residues (in other words, specific for a deletion mutant in these four residues). Examples of such oligonucleotides include the following nucleotide sequence: AAACATGACAGATAGCTTTGCAAAT (corresponding to residues 284-309 of SEQ ID NO: 1), and sequences complementary therewith, wherein the underlined four nucleotides can be left out to create an oligonucleotide specific for the disclosed gene truncation mutation.

Example 6: Detecting *mdr1* Mutations

The truncation mutation at nucleotide residue 294-297 of SEQ ID NO: 1 of canine *mdr1* (the first position of which encodes amino acid residue 75 of P-gp, SEQ ID NO: 2) can be detected by a variety of techniques. These techniques include allele-specific oligonucleotide hybridization (ASOH) (Stoneking *et al.*, *Am. J. Hum. Genet.* 48:370-382, 1991), which involves hybridization of oligonucleotide probes to the sequence, stringent washing, and signal detection. Other applicable methods include techniques that incorporate more robust scoring of hybridization. Examples of these procedures include the ligation chain reaction (ASOH plus selective ligation and amplification), as

disclosed in Wu and Wallace (*Genomics* 4:560-569, 1989); mini-sequencing (ASOH plus a single base extension) as discussed in Syvanen (*Meth. Mol. Biol.* 98:291-298, 1998); and the use of DNA chips (miniaturized ASOH with multiple oligonucleotide arrays) as disclosed in Lipshutz *et al.* (*BioTechniques* 19:442-447, 1995). Alternatively, ASOH with single- or dual- labeled probes can be merged with PCR, as in the 5'-exonuclease assay (Heid *et al.*, *Genome Res.* 6:986-994, 1996), or with molecular beacons (as in Tyagi and Kramer, *Nat. Biotechnol.* 14:303-308, 1996).

Another technique is dynamic allele-specific hybridization (DASH), which involves dynamic heating and coincident monitoring of DNA denaturation, as disclosed by Howell *et al.* (*Nat. Biotech.* 17:87-88, 1999). A target sequence is amplified (*e.g.*, by PCR) using one biotinylated primer. The biotinylated product strand is bound to a streptavidin-coated microtiter plate well (or other suitable surface), and the non-biotinylated strand is rinsed away with alkali wash solution. An oligonucleotide probe, specific for one allele (*e.g.*, the wild-type allele), is hybridized to the target at low temperature. This probe forms a duplex DNA region that interacts with a double strand-specific intercalating dye. When subsequently excited, the dye emits fluorescence proportional to the amount of double-stranded DNA (probe-target duplex) present. The sample is then steadily heated while fluorescence is continually monitored. A rapid fall in fluorescence indicates the denaturing temperature of the probe-target duplex. Using this technique, a single-base mismatch between the probe and target results in a significant lowering of melting temperature (T_m) that can be readily detected.

A variety of other techniques can be used to detect the polymorphisms in DNA. Merely by way of example, see U.S. Patent Nos. 4,666,828; 4,801,531; 5,110,920; 5,268,267; 5,387,506; 5,691,153; 5,698,339; 5,736,330; 5,834,200; 5,922,542; and 5,998,137 for such methods.

Example 7: Expression of P-gp

The expression and purification of proteins, such as the P-gp, can be performed using standard laboratory techniques. After expression, purified P-gp may be used for functional analyses, antibody production, diagnostics, and patient therapy. Furthermore, the DNA sequence of the *mdr1* cDNA can be manipulated in studies to understand the expression of the gene and the function of its product. Mutant forms of the canine *mdr1* gene may be isolated based upon information contained herein, and may be studied in order to detect alteration in expression patterns in terms of relative quantities, tissue specificity, molecular weight, stability, and functional properties of the encoded mutant P-gp protein. Partial or full-length cDNA sequences, which encode for the subject protein, may be ligated into bacterial expression vectors. Methods for expressing large amounts of protein from a cloned gene introduced into *Escherichia coli* (*E. coli*) may be utilized for the purification, localization and functional analysis of proteins. For example, fusion proteins consisting of amino terminal peptides encoded by a portion of the *E. coli lacZ* or *trpE* gene linked to P-gp proteins may be used to prepare polyclonal and monoclonal antibodies against these proteins. Thereafter, these antibodies may be used to purify proteins by immunoaffinity chromatography, in diagnostic assays to

quantitate the levels of protein and to localize proteins in tissues and individual cells by immunofluorescence.

Intact native protein may also be produced in *E. coli* in large amounts for functional studies. Methods and plasmid vectors for producing fusion proteins and intact native proteins in bacteria are described in Sambrook *et al.* (In *Molecular Cloning: A Laboratory Manual*, Ch. 17, CSHL, New York, 1989). Such fusion proteins may be made in large amounts, are easy to purify, and can be used to elicit antibody response. Native proteins can be produced in bacteria by placing a strong, regulated promoter and an efficient ribosome-binding site upstream of the cloned gene. If low levels of protein are produced, additional steps may be taken to increase protein production; if high levels of protein are produced, purification is relatively easy. Suitable methods are presented in Sambrook *et al.* (In *Molecular Cloning: A Laboratory Manual*, CSHL, New York, 1989) and are well known in the art. Often, proteins expressed at high levels are found in insoluble inclusion bodies. Methods for extracting proteins from these aggregates are described by Sambrook *et al.* (In *Molecular Cloning: A Laboratory Manual*, Ch. 17, CSHL, New York, 1989). Vector systems suitable for the expression of *lacZ* fusion genes include the pUR series of vectors (Ruther and Muller-Hill, *EMBO J.* 2:1791, 1983), pEX1-3 (Stanley and Luzio, *EMBO J.* 3:1429, 1984) and pMR100 (Gray *et al., Proc. Natl. Acad. Sci. USA* 79:6598, 1982). Vectors suitable for the production of intact native proteins include pKC30 (Shimatake and Rosenberg, *Nature* 292:128, 1981), pKK177-3 (Amann and Brosius, *Gene* 40:183, 1985) and pET-3 (Studiar and Moffatt, *J. Mol. Biol.* 189:113, 1986). P-gp fusion proteins may be isolated from protein gels, lyophilized, ground into a powder and used as an antigen. The DNA sequence can also be transferred from its existing context to other cloning vehicles, such as other plasmids, bacteriophages, cosmids, animal viruses and yeast artificial chromosomes (YACs) (Burke *et al., Science* 236:806-812, 1987). These vectors may then be introduced into a variety of hosts including somatic cells, and simple or complex organisms, such as bacteria, fungi (Timberlake and Marshall, *Science* 244:1313-1317, 1989), invertebrates, plants (Gasser and Fraley, *Science* 244:1293, 1989), and animals (Pursel *et al., Science* 244:1281-1288, 1989), which cell or organisms are rendered transgenic by the introduction of the heterologous *mdr1* cDNA.

For expression in mammalian cells, the cDNA sequence may be ligated to heterologous promoters, such as the simian virus (SV) 40 promoter in the pSV2 vector (Mulligan and Berg, *Proc. Natl. Acad. Sci. USA* 78:2072-2076, 1981), and introduced into cells, such as monkey COS-1 cells (Gluzman, *Cell* 23:175-182, 1981), to achieve transient or long-term expression. The stable integration of the chimeric gene construct may be maintained in mammalian cells by biochemical selection, such as neomycin (Southern and Berg, *J. Mol. Appl. Genet.* 1:327-341, 1982) and mycophenolic acid (Mulligan and Berg, *Proc. Natl. Acad. Sci. USA* 78:2072-2076, 1981).

DNA sequences can be manipulated with standard procedures such as restriction enzyme digestion, fill-in with DNA polymerase, deletion by exonuclease, extension by terminal deoxynucleotide transferase, ligation of synthetic or cloned DNA sequences, site-directed sequence-

alteration via single-stranded bacteriophage intermediate or with the use of specific oligonucleotides in combination with PCR.

The cDNA sequence (or portions derived from it) or a mini gene (a cDNA with an intron and its own promoter) may be introduced into eukaryotic expression vectors by conventional techniques. These vectors are designed to permit the transcription of the cDNA in eukaryotic cells by providing regulatory sequences that initiate and enhance the transcription of the cDNA and ensure its proper splicing and polyadenylation. Vectors containing the promoter and enhancer regions of the SV40 or long terminal repeat (LTR) of the Rous Sarcoma virus and polyadenylation and splicing signal from SV40 are readily available (Mulligan *et al., Proc. Natl. Acad. Sci. USA* 78:1078-2076, 1981; Gorman *et al., Proc. Natl. Acad. Sci. USA* 78:6777-6781, 1982). The level of expression of the cDNA can be manipulated with this type of vector, either by using promoters that have different activities. For example, the baculovirus pAC373 can express cDNAs at high levels in *S. frugiperda* cells (Summers and Smith, In *Genetically Altered Viruses and the Environment*, Fields *et al.* (Eds.) 22:319-328, CSHL Press, Cold Spring Harbor, New York, 1985) or by using vectors that contain promoters amenable to modulation, for example, the glucocorticoid-responsive promoter from the mouse mammary tumor virus (Lee *et al., Nature* 294:228, 1982). The expression of the cDNA can be monitored in the recipient cells 24 to 72 hours after introduction (transient expression).

In addition, some vectors contain selectable markers such as the *gpt* (Mulligan and Berg, *Proc. Natl. Acad. Sci. USA* 78:2072-2076, 1981) or *neo* (Southern and Berg, *J. Mol. Appl. Genet.* 1:327-341, 1982) bacterial genes. These selectable markers permit selection of transfected cells that exhibit stable, long-term expression of the vectors (and therefore the cDNA). The vectors can be maintained in the cells as episomal, freely replicating entities by using regulatory elements of viruses such as papilloma (Sarver *et al., Mol. Cell Biol.* 1:486, 1981) or Epstein-Barr (Sugden *et al., Mol. Cell Biol.* 5:410, 1985). Alternatively, one also can produce cell lines that have integrated the vector into genomic DNA. Both of these types of cell lines produce the gene product on a continuous basis. One can also produce cell lines that have amplified the number of copies of the vector (and therefore of the cDNA as well) to create cell lines that can produce high levels of the gene product (Alt *et al., J. Biol. Chem.* 253:1357, 1978).

The transfer of DNA into eukaryotic, in particular human or other mammalian cells, is now a conventional technique. The vectors are introduced into the recipient cells as pure DNA (transfection) by, for example, precipitation with calcium phosphate (Graham and van der Eb, *Virology* 52:456-467, 1973) or strontium phosphate (Brash *et al., Mol. Cell Biol.* 7:2013, 1987), electroporation (Neumann *et al., EMBO J* 1:841, 1982), lipofection (Felgner *et al., Proc. Natl. Acad. Sci USA* 84:7413, 1987), DEAE dextran (McCuthan *et al., J. Natl. Cancer Inst.* 41:351, 1968), microinjection (Mueller *et al., Cell* 15:579, 1978), protoplast fusion (Schafner, *Proc. Natl. Acad. Sci. USA* 77:2163-2167, 1980), or pellet guns (Klein *et al., Nature* 327:70, 1987). Alternatively, the cDNA, or fragments thereof, can be introduced by infection with virus vectors. Systems are developed that use, for example, retroviruses (Bernstein *et al., Gen. Engr.* 7:235, 1985), adenoviruses

(Ahmad *et al., J. Virol.* 57:267, 1986), or Herpes virus (Spaete *et al., Cell* 30:295, 1982). P-gp encoding sequences also can be delivered to target cells *in vitro* via non-infectious systems, for instance liposomes.

These eukaryotic expression systems can be used for studies of P-gp encoding nucleic acids and mutant forms of these molecules, the P-gp protein and mutant forms of this protein.

Using the above techniques, the expression vectors containing the *mdr1* gene sequence or cDNA, or fragments or variants or mutants thereof, can be introduced into canine cells, mammalian cells from other species, or non-mammalian cells as desired. The choice of cell is determined by the purpose of the introduction. For example, monkey COS cells (Gluzman, *Cell* 23:175-182, 1981) that produce high levels of the SV40 T antigen and permit the replication of vectors containing the SV40 origin of replication may be used. Similarly, Chinese hamster ovary (CHO), mouse NIH 3T3 fibroblasts or canine fibroblasts or lymphoblasts may be used.

This disclosure thus encompasses recombinant vectors that comprise all or part of the *mdr1* gene or cDNA sequences, for expression in a suitable host. The *mdr1* DNA is operatively linked in the vector to an expression control sequence in the recombinant DNA molecule so that the P-gp polypeptide can be expressed. The expression control sequence may be selected from the group consisting of sequences that control the expression of genes of prokaryotic or eukaryotic cells and their viruses and combinations thereof. The expression control sequence may be specifically selected from the group consisting of the *lac* system, the *trp* system, the *tac* system, the *trc* system, major operator and promoter regions of phage lambda, the control region of fd coat protein, the early and late promoters of SV40, promoters derived from polyoma, adenovirus, retrovirus, baculovirus and simian virus, the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, the promoter of the yeast alpha-mating factors and combinations thereof.

The host cell, which may be transfected with a vector described herein, may be selected from the group consisting of *E. coli*, *Pseudomonas*, *Bacillus subtilis*, *Bacillus stearothermophilus* or other bacilli; other bacteria; yeast; fungi; insect; mouse or other animal; or plant hosts; or human or canine tissue cells.

It is appreciated that, for mutant or variant *mdr1* DNA sequences, similar systems are employed to express and produce the mutant product. In addition, fragments of the P-gp protein can be expressed essentially as detailed above. Such fragments include individual P-gp protein domains or sub-domains, as well as shorter fragments such as peptides. P-gp protein fragments having therapeutic properties may be expressed in this manner also.

Example 8: Production of P-gp Protein Specific Binding Agents

Monoclonal or polyclonal antibodies may be produced to either the normal P-gp or mutant forms (*e.g.*, truncations) of this protein. Optimally, antibodies raised against these proteins or peptides would specifically detect the protein or peptide with which the antibodies are generated.

That is, an antibody generated to the P-gp or a fragment thereof would recognize and bind the P-gp and would not substantially recognize or bind to other proteins found in human cells.

The determination that an antibody specifically detects P-gp is made by any one of a number of standard immunoassay methods; for instance, the Western blotting technique (Sambrook *et al.,* In *Molecular Cloning: A Laboratory Manual*, CSHL, New York, 1989). To determine that a given antibody preparation (such as one produced in a mouse) specifically detects P-gp by Western blotting, total cellular protein is extracted from human cells (for example, lymphocytes) and electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel. The proteins are then transferred to a membrane (for example, nitrocellulose) by Western blotting, and the antibody preparation is incubated with the membrane. After washing the membrane to remove non-specifically bound antibodies, the presence of specifically bound antibodies is detected by the use of an anti-mouse antibody conjugated to an enzyme such as alkaline phosphatase. Application of an alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium results in the production of a dense blue compound by immunolocalized alkaline phosphatase. Antibodies that specifically detect P-gp will, by this technique, be shown to bind to P-gp band (which will be localized at a given position on the gel determined by its molecular weight). Non-specific binding of the antibody to other proteins may occur and may be detectable as a weak signal on the Western blot. The non-specific nature of this binding will be recognized by one skilled in the art by the weak signal obtained on the Western blot relative to the strong primary signal arising from the specific antibody-P-gp binding.

Substantially pure P-gp or protein fragments (peptides) suitable for use as an immunogen may be isolated from transfected or transformed cells as described above. Concentration of protein or peptide in the final preparation is adjusted, for example, by concentration on an Amicon filter device, to the level of a few micrograms per milliliter. Monoclonal or polyclonal antibody to the protein can then be prepared using one of the following techniques.

A. Monoclonal Antibody Production by Hybridoma Fusion

Monoclonal antibody to epitopes of P-gp, or specifically to the truncation protein identified and isolated as described can be prepared from murine hybridomas according to the classical method of Kohler and Milstein (*Nature* 256:495-497, 1975) or derivative methods thereof. Briefly, a mouse is repetitively inoculated with a few micrograms of the selected protein over a period of a few weeks. The mouse is then sacrificed, and the antibody-producing cells of the spleen isolated. The spleen cells are fused by means of polyethylene glycol with mouse myeloma cells, and the excess un-fused cells destroyed by growth of the system on selective media comprising aminopterin (HAT media). The successfully fused cells are diluted and aliquots of the dilution placed in wells of a microtiter plate where growth of the culture is continued. Antibody-producing clones are identified by detection of antibody in the supernatant fluid of the wells by immunoassay procedures, such as ELISA, as originally described by Engvall (*Meth. Enzymol.* 70:419-439, 1980), and derivative

methods thereof. Selected positive clones can be expanded and their monoclonal antibody product harvested for use. Detailed procedures for monoclonal antibody production are described in Harlow and Lane (*Antibodies, A Laboratory Manual*, CSHL, New York, 1988).

B. Polyclonal Antibody Production by Immunization

Polyclonal antiserum containing antibodies to heterogeneous epitopes of a single protein can be prepared by immunizing suitable animals with the expressed protein (Example 7), which can be unmodified or modified to enhance immunogenicity. Effective polyclonal antibody production is affected by many factors related both to the antigen and the host species. For example, small molecules tend to be less immunogenic than others and may require the use of carriers and adjuvant. Also, host animals vary in response to site of inoculations and dose, with either inadequate or excessive doses of antigen resulting in low titer antisera. Small doses (ng level) of antigen administered at multiple intradermal sites appear to be most reliable. An effective immunization protocol for rabbits can be found in Vaitukaitis *et al.* (*J. Clin. Endocrinol. Metab.* 33:988-991, 1971).

Booster injections can be given at regular intervals, and antiserum harvested when antibody titer thereof, as determined semi-quantitatively, for example, by double immunodiffusion in agar against known concentrations of the antigen, begins to fall. See, for example, Ouchterlony *et al.* (In *Handbook of Experimental Immunology*, Wier, D. (ed.) chapter 19. Blackwell, 1973). Plateau concentration of antibody is usually in the range of about 0.1 to 0.2 mg/ml of serum (about 12 μM). Affinity of the antisera for the antigen is determined by preparing competitive binding curves, as described, for example, by Fisher (*Manual of Clinical Immunology*, Ch. 42, 1980).

C. Antibodies Raised against Synthetic Peptides

A third approach to raising antibodies against P-gp or peptides is to use one or more synthetic peptides synthesized on a commercially available peptide synthesizer based upon the predicted amino acid sequence of P-gp or peptide. Polyclonal antibodies can be generated by injecting these peptides into, for instance, rabbits.

D. Antibodies Raised by Injection of P-gp Encoding Sequence

Antibodies may be raised against P-gp proteins and peptides by subcutaneous injection of a DNA vector that expresses the desired protein or peptide, or a fragment thereof, into laboratory animals, such as mice. Delivery of the recombinant vector into the animals may be achieved using a hand-held form of the Biolistic system (Sanford *et al., Particulate Sci. Technol.* 5:27-37, 1987) as described by Tang *et al.* (*Nature* 356:152-154, 1992). Expression vectors suitable for this purpose may include those that express the P-gp encoding sequence (*mdr1* gene or cDNA, for instance) under the transcriptional control of either the human β-actin promoter or the cytomegalovirus (CMV) promoter.

Antibody preparations prepared according to these protocols are useful in quantitative immunoassays which determine concentrations of antigen-bearing substances in biological samples;

they are also used semi-quantitatively or qualitatively to identify the presence of antigen in a biological sample; or for immunolocalization of P-gp.

For administration to specific animal subjects (such as human or canine individuals), antibodies, *e.g.*, P-gp specific monoclonal antibodies, can be adapted to be more effective in the target organism by methods known in the art. By way of example, antibodies with a desired binding specificity can be commercially humanized (Scotgene, Scotland, UK; Oxford Molecular, Palo Alto, CA).

Example 9: Protein-Based Detection

An alternative method of detecting sensitivity to ivermectin and related drugs (those that are kept from crossing the blood-brain barrier by P-gp) is to examine the level or molecular weight (apparent size, *e.g.*, on after SDS-PAGE with or without immunodetection) of P-gp in the cells of an individual. These diagnostic tools would be useful for detecting reduced levels of P-gp that result from, for example, mutations in the promoter regions of the *mdr1* gene or mutations within the coding region of the gene that produced truncated, non-functional or unstable polypeptides, as well as from deletions of a portion of or the entire *mdr1* gene.

Localization and/or coordinated *mdr1* expression (temporally or spatially) can also be examined using known techniques, such as isolation and comparison of P-gp from cell or tissue specific, or time specific, samples. The determination of reduced or increased P-gp levels, in comparison to such expression in a control cell (*e.g.*, normal, as in taken from an individual not exhibiting sensitivity to ivermectin or another neurotoxin), would be an alternative or supplemental approach to the direct determination of *mdr1* gene mutation (*e.g.*, truncation mutation) status by the methods outlined above and equivalents.

The availability of antibodies specific to P-gp will facilitate the detection, measurement (*e.g.*, molecular weight determination) and quantitation of cellular P-gp by one of a number of immunoassay methods which are well known in the art and are presented in Harlow and Lane (*Antibodies, A Laboratory Manual*, CSHL, New York, 1988). Methods of constructing such antibodies are discussed above, in Example 8.

Any standard immunoassay format (*e.g.*, ELISA, Western blot, or RIA assay) can be used to measure P-gp polypeptide or protein levels; comparison is to wild-type (normal) P-gp, and an alteration in P-gp polypeptide may be indicative of an abnormal biological condition regarding resistance to potential neurotoxins, such as ivermectin. Immunohistochemical techniques may also be utilized for P-gp polypeptide or protein detection. For example, a tissue sample may be obtained from a subject, and a section stained for the presence of P-gp using a P-gp specific binding agent (*e.g.*, anti-P-gp antibody) and any standard detection system (*e.g.*, one which includes a secondary antibody conjugated to horseradish peroxidase). General guidance regarding such techniques can be found in, *e.g.*, Bancroft and Stevens (*Theory and Practice of Histological Techniques*, Churchill

Livingstone, 1982) and Ausubel *et al.* (*Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1998).

For the purposes of quantifying or determining the estimated molecular weight of P-gp, a biological sample of the subject (which can be any animal, for instance a dog or a human), which sample includes cellular proteins, is required. Such a biological sample may be obtained from body cells, such as those present in peripheral blood, urine, saliva, tissue biopsy, amniocentesis samples, surgical specimens and autopsy material. Quantitation and/or measurement of P-gp can be achieved by immunoassay and compared to level and apparent size of the protein found in control cells (*e.g.*, healthy, as in from an individual known not to have ivermectin sensitivity). A significant (*e.g.*, 10% or greater) reduction in the amount of P-gp in the cells of a test subject compared to the amount of P-gp found in normal cells, or a substantial reduction in the apparent molecular weight of the P-gp (*e.g.*, as would be apparent with a truncation mutation) could be taken as an indication that the subject may have deletions or mutations in the *mdr1* gene. Deletion and/or mutation of or within the *mdr1*-encoding sequence, and substantial under-expression of P-gp, may be indicative of altered sensitivity to ivermectin and other drugs that are usually kept from crossing the blood-brain barrier by P-gp.

Merely by way of example, canine P-gp can be analyzed as described in Mealey *et al.*, *Cancer Letters* 126:187-192, 1998. For instance, immunoblotting has been carried out using the following procedure:

Cells were harvested by trypsinization, washed with DPBS and solubilized in tumor solubilization buffer (TSB, 50 mM Tris-HCl (pH 6.8), 50 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 2% CHAPS, 0.1 mM leupeptin, 0.2 mM phenylmethyl-sulfonylfluoride and 10 mM dithiothreitol). Insoluble complexes were cleared by a 5-minute spin (1500 revolutions/minute) and the soluble protein was collected for quantitation using a modified Lowry technique (Lowry, *J. Biol. Chem.* 193:265-275, 1951).

Protein samples were separated by SDS-PAGE and electroblotted onto an Immobilon-P™ membrane (Millipore, Bedford, MA). Membranes were washed with blotto buffer (50 mM Tris-HCl, 2 mM $CaCl_2$, 80 mM NaCl, 5% non-fat dry milk, 0.2% Nonidet P-40 and 0.03% sodium azide) for one hour at 25°C and then incubated (25°C for 16 hours) with a murine anti-human P-glycoprotein monoclonal antibody (C219; Signet, Dedham, MA). Actin was subsequently detected using a monoclonal anti-actin antibody (ICN Immunobiologicals, Costa Mesa, CA). Membranes were washed in fresh blotto (non-fat milk) buffer and incubated with the appropriate alkaline phosphatase-labeled secondary antibody. Membranes were washed with buffer A (50 mM Tris-HCl, 2 mM $CaCl_2$ and 80 mM NaCl) and developed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate with an alkaline-phosphatase conjugate substrate kit (BioRad, Hercules, CA). The color reaction was terminated by washing in distilled water. The resulting bands were scanned with a Visage 110 camera-based densitometer (Bio-Image, Ann Arbor, MN) and analyzed using whole band software. Integrated intensity signals for P-glycoprotein can be normalized to those of a protein the level of which is not expected to change under the experimental conditions.

Example 10: Differentiation of Individuals Homozygous versus Heterozygous for the Polymorphism(s)

As will be appreciated, the oligonucleotide ligation assay (OLA), as described at Nickerson *et al.* (*Proc. Natl. Acad. Sci. USA* 87:8923-8927, 1990), allows the differentiation between individuals who are homozygous versus heterozygous for the herein-described frame-shift truncation mutation in *mdr1*. This feature allows one to rapidly and easily determine whether an individual is homozygous for at least a neurotoxin sensitivity-linked polymorphism, which condition can result in neurotoxicosis and possible death when an individual is administered an otherwise safe drug dosage. Alternatively, OLA can be used to determine whether a subject is homozygous for either of these polymorphisms.

As an example of the OLA assay, when carried out in microtiter plates, one well is used for the determination of the presence of the *mdr1* allele that contains the herein-described frame-shift truncation mutation, and a second well is used for the determination of the presence of the *mdr1* wild-type allele. Thus, the results for an individual who is heterozygous for the polymorphism will show a signal in each of the "truncated" and wild-type wells, and an individual who is homozygous for one allele or the other will show a signal only in the corresponding well.

Likewise, truncation itself can be used to detect heterogeneity in the P-gp protein. Because truncation leads to production of a shorter (and therefore "lighter") protein product, Western analysis can be used to distinguish between heterozygous and homozygous individuals, as well as between homozygous truncated (and therefore sensitive) and wild-type (resistant) individuals.

Example 11: Kits

Kits are provided which contain the necessary reagents for determining the presence or absence of polymorphism(s) in a P-gp-encoding sequence, such as probes or primers specific for the *mdr1* gene. Such kits can be used with the methods described herein to determine whether an individual is likely to be sensitive to ivermectin and other drugs that are usually kept from crossing the blood-brain barrier by P-gp (such as those listed in Table II).

The provided kits may also include written instructions. The instructions can provide calibration curves or charts to compare with the determined (*e.g.*, experimentally measured) values.

Kits are also provided to determine altered (*e.g.*, lowered) expression of mRNA (*i.e.*, containing probes) or P-gp protein (*i.e.*, containing antibodies or other P-gp-specific binding agents), as well as truncated P-gp.

A. Kits for Detecting *mdr1* Nucleic Acid Mutations

The oligonucleotide probes and primers disclosed herein can be supplied in the form of a kit, for use in detection of ivermectin sensitivity in a subject. In such a kit, an appropriate amount of one or more of the oligonucleotides is provided in one or more containers. The oligonucleotides may be

provided suspended in an aqueous or other solution, or as a freeze-dried or lyophilized powder, for instance. The container(s) in which the oligonucleotide(s) are supplied can be any conventional container that is capable of holding the supplied form, for instance, microfuge tubes, ampoules, or bottles. In some applications, pairs of primers may be provided in pre-measured single use amounts in individual, typically disposable, tubes or equivalent containers. With such an arrangement, the sample to be tested for the presence of an *mdr1* polymorphism can be added to the individual tubes and amplification or other laboratory manipulation carried out directly.

The amount of each oligonucleotide supplied in the kit can be any appropriate amount, depending for instance on the market to which the product is directed. For instance, if the kit is adapted for research or clinical use, the amount of each oligonucleotide primer provided would likely be an amount sufficient to prime several *in vitro* nucleic acid amplification reactions. Those of ordinary skill in the art know the amount of oligonucleotide primer that is appropriate for use in a single amplification reaction. General guidelines may for instance be found in Innis et al. (*PCR Protocols, A Guide to Methods and Applications*, Academic Press, Inc., San Diego, CA, 1990), Sambrook *et al.* (In *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989), and Ausubel *et al.* (In *Current Protocols in Molecular Biology*, Greene Publ. Assoc. and Wiley-Intersciences, 1992).

A kit may include more than two primers, in order to facilitate the *in vitro* amplification of *mdr1* sequences, for instance the *mdr1* gene or the 5'- or 3'-flanking region thereof.

In some embodiments, kits may also include the reagents necessary to carry out nucleotide amplification reactions, including, for instance, DNA sample-preparation reagents, appropriate buffers *(e.g.,* polymerase buffer), salts (*e.g.*, magnesium chloride), and deoxyribonucleotides (dNTPs).

Kits may include either labeled or unlabeled oligonucleotide probes for use in detection of *mdr1* polymorphism(s). In certain embodiments, these probes will be specific for a potential polymorphism that may be present in the target sequence. The appropriate sequences for such a probe will be any sequence that includes all or part of the identified polymorphic site, particularly nucleotide positions 294 through 297 of the canine *mdr1* gene, such that the sequence the probe is complementary to the truncation polymorphic site and a portion of the surrounding *mdr1* sequence. An oligonucleotide including the sequence AAACATGACAGATAGCTTTGCAAAT (corresponding to residues 284-309 of SEQ ID NO: 1) exemplifies such a sequence, and a probe useful for disclosed methods could comprise this sequence. Alternatively, an example of a probe specific for detecting the ivermectin sensitivity allele of *mdr1* may include the following sequence: AAACATGACAGCTTTGCAAAT (also corresponding to residues 284-309 of SEQ ID NO: 1, but with residues 294-297 removed).

It also may be advantageous to provide in the kit one or more control sequences for use in the amplification reactions. The design of appropriate positive and negative control sequences is well known to one of ordinary skill in the appropriate art.

B. Kits for Detection of *mdr1* mRNA

Kits similar to those disclosed above for the detection of *mdr1* polymorphisms directly can be used to detect *mdr1* mRNA expression, such as over- or under-expression or expression of a truncated form. Such kits include an appropriate amount of one or more oligonucleotide primers for use in, for instance, reverse transcription nucleic acid amplification reactions (*e.g.*, RT-PCR), similarly to those oligonucleotides described above with art-obvious modifications for use with RNA amplification.

In some embodiments, kits for detection of altered expression of *mdr1* mRNA may also include some or all of the reagents necessary to carry out RT-PCR or other *in vitro* amplification reactions, for instance, RNA sample preparation reagents (including *e.g.*, an RNase inhibitor), appropriate buffers (*e.g.*, polymerase buffer), salts (*e.g.*, magnesium chloride), and deoxyribonucleotides (dNTPs). Written instructions also may be included.

Such kits may in addition include either labeled or unlabeled oligonucleotide probe(s) for use in detection of the *in vitro* amplified target sequences. The appropriate sequences for such a probe will be any sequence that falls between the annealing sites of the two provided oligonucleotide primers, such that the sequence the probe is complementary to is amplified during the *in vitro* amplification reaction. In certain embodiments, these probes will be specific for a potential polymorphism that may be present in the amplified target sequences.

It also may be advantageous to provide in the kit one or more control sequences for use in the RT-PCR reactions. The design of appropriate positive and negative control sequences is well known to one of ordinary skill in the appropriate art.

Alternatively, kits may be provided with the necessary reagents to carry out quantitative or semi-quantitative Northern analysis of *mdr1* mRNA. Such kits include, for instance, at least one *mdr1*-specific oligonucleotide for use as a probe. This oligonucleotide may be labeled in any conventional way, including with a selected radioactive isotope, enzyme substrate, co-factor, ligand, chemiluminescent or fluorescent agent, hapten, or enzyme. In certain embodiments, such probes will be specific for a potential polymorphism (*e.g.*, a truncation mutation) that may be present in the target sequences.

C. Kits For Detection of P-gp Expression

Kits for the detection of P-gp protein expression (such as over- or under-expression), and particularly changes in the apparent molecular weight of expressed P-gp, are also encompassed herein. Such kits may include at least one target-protein-specific binding agent (*e.g.*, a polyclonal or monoclonal antibody or antibody fragment that specifically recognizes P-gp) and may include at least one control (such as a determined amount of P-gp, with a defined molecular weight or apparent size, or a sample containing a determined amount of P-gp). The P-gp-protein-specific binding agent and control may be contained in separate containers.

P-gp expression detection kits may also include a means for detecting P-gp:binding agent complexes, for instance the agent may be detectably labeled. If the binding agent is not labeled, it

may be detected by second antibodies or protein A for example, which components may also be provided in some kits in one or more separate containers. Such detection techniques are known.

Additional components in specific kits may include instructions for carrying out the assay. Instructions will allow the tester to determine whether P-gp expression levels are elevated, and/or whether the expressed P-gp has an altered molecular weight compared to wild-type P-gp. Reaction vessels and auxiliary reagents such as chromogens, buffers, enzymes, etc. may also be included in the kits.

D. Kits for Detection of Homozygous versus Heterozygous Allelism

Also provided are kits that allow differentiation between individuals who are homozygous versus heterozygous for a truncation mutation in the *mdr1* gene. Certain examples of such kits provide the materials for performing oligonucleotide ligation assays (OLA), as described by Nickerson *et al., Proc. Natl. Acad. Sci. USA* 87:8923-8927, 1990. In specific embodiments, these kits contain one or more microtiter plate assays, designed to detect polymorphism(s) in the *mdr1* sequence of a subject, as described herein.

In other examples of such kits, materials are provided for examining the size of the P-gp expressed by an individual, for instance components for carrying out a Western analysis or other immunological assay.

Additional components in some of these kits may include instructions for carrying out the assay. Instructions will allow the tester to determine whether an *mdr1* truncation mutation allele is homozygous or heterozygous, either through examination of nucleic acid molecules or protein. Reaction vessels and auxiliary reagents such as chromogens, buffers, enzymes, etc. may also be included in the kits.

It may also be advantageous to provide in the kit one or more control sequences for use in the OLA or immunoassay reactions. The design of appropriate positive and negative control molecules is well known to one of ordinary skill in the appropriate art.

Example 12: Animal Model

A large number of P-gp substrates are routinely prescribed in humans, including HIV-1 protease inhibitors and many chemotherapeutic agents. Animals (*e.g.*, Collies) possessing a polymorphism of the *mdr1* gene, for instance, the truncation mutation described herein, can serve as a useful model for studying the effects of P-glycoprotein substrates on humans with *mdr1* polymorphisms. In addition, these animals can serve as models for studying the effects of compounds that interact with P-gp. They are also useful to study pharmacologic inhibition of P-gp, for instance in order to identify or characterize modulators of P-gp transport activity that may be useful to increase (or decrease, or regulate) drug absorption in or distribution to one or more tissues in a subject.

Use of the animals identified herein (particularly Collies) possessing a naturally-occurring polymorphism of the *mdr1* gene avoids confounding effects attributable to producing *mdr1* mutations

through genetic engineering, and is expected to result in better acceptance as a research model by both the research community and society.

Using methods described herein, individual animals are identified as being heterozygous or homozygous for an *mdr1* truncation, and these animals are used as subjects for animal model studies. In specific examples, a drug of interest (or drug candidate or other compound or mixture thereof) is administered to Collies possessing a polymorphism of MDR-1, and the effects are monitored systemically, for instance, or in particular tissues. Routes of administration include but are not limited to oral and parenteral routes, such as intravenous (iv), intraperitoneal (ip), rectal, topical, ophthalmic, nasal, and transdermal.

Effective doses of the compound(s) of interest can be determined by one of ordinary skill in the art, and may be tailored to the specific experiments being run. In some embodiments, the compound is administered with a goal of achieving tissue concentrations that are at least as high as the IC_{50} of the compounds(s) tested. An example of such a dosage range is 0.1 to 200 mg/kg body weight. The specific dose level and frequency of dosage for any particular subject may be varied and will depend upon a variety of factors, including the activity of the specific compound, the metabolic stability and length of action of that compound, the age, body weight, mode and time of administration, and the rate of excretion of the compound.

Systemic effects of the compound of interest can be monitored in the animal following its administration. For example, neurological symptoms linked to a drug passing across one or more cellular membranes via P-gp can include salivation, vomiting, confusion, ataxia, tremors, seizure-like activity, recumbency, non-responsiveness, and coma. Alternatively, drug or other compound levels in the blood, or biochemical changes in the gastrointestinal tract, kidneys, nervous system, liver, tumor cells, or other tissues, can be monitored.

The animal model can also be used to identify and/or characterize test compounds known to, or expected to, modulate the activity of P-gp. In examples of such methods, the animals are treated (*e.g.*, concurrently or sequentially) with both the test compound and a compound known to be transported by P-gp (the effector molecule). The animals are then monitored to detect one or more effects caused by the effector compound, and the level of effect can be compared to animals that were not treated with the test compound (or which received a different treatment regimen). Depending on the effector molecule used, different biological characteristics of the animal, or a tissue or cell within the animal, can be monitored to determine whether (and to what extent) the test compound influences P-gp interaction with the effector. Depending on whether the test molecule increases or decreases P-gp transport of the effector, the test molecule is identified as an agonist or antagonist of P-gp activity and can be selected for further characterization.

Example 13: Cultured Cells

Cells or tissues from animals (*e.g.*, Collies) possessing a polymorphism of the *mdr1* gene, for instance, the truncation mutation described herein, also can serve as useful model systems for

studying the effects of P-gp interacting molecules. In addition, cells and tissues from animals possessing a polymorphism of the *mdr1* gene can serve as models for studying pharmacologic inhibition, stimulation, and/or regulation of P-gp.

In some examples of such cell-based models, cells from an animal carrying an *mdr1* truncation as described herein are transformed (stably or transiently) with an *mdr1* construct, for instance which comprises known (*e.g.*, engineered) or unknown mutations (*e.g.*, point mutations such as a naturally occurring polymorphism, for instance polymorphisms identified in human *mdr1*). Such cellular expression systems enable examination of the activity of the mutant *mdr1* construct in a defined large mammal background.

P-gp is expressed in many tissues throughout the mammalian body, including the epithelium of the gastrointestinal tract, renal-tubular epithelium, brain capillary endothelial cells, biliary tubular epithelial cells, and at the plasma membrane in many tumor cells. In some examples, cells used for the *in vitro* cell system are selected from a specific tissue or cell type, so that tissue or cell specific effects of P-gp transport can be studied. In carrying out such methods, specific tissues are isolated from canines (*e.g.*, Collies) possessing a polymorphism of the *mdr1* gene, such as the truncation described herein. Though methods are widely known for isolating cells and tissues of specific types, the following procedures are provided as specific examples and can be used to isolate specific cells from canine samples:

(a) Canine intestinal epithelial cells can be isolated and cultured as described in Koop and Buchan, *Gastroenterology* 102: 28-34, 1992.

(b) Canine brain microvessel endothelial cells can be isolated and cultured as described in Drewes *et al.*, *Brain Research Bulletin* 21: 771-776, 1988.

(c) Canine renal-tubular cells can be isolated and cultured as described in Hamada *et al.*, *Nephron.* 68: 104-111, 1994.

(d) Canine hepatocytes can be isolated and cultured as described in Lu and Li, *Chem. Biol. Interact.* 143: 271-281, 2001, Amacher and Martin, *Fundam. Appl Toxicol.* 40:256-263, 1997, or Placidi *et al.*, *Drug Metab. Dispos.* 25: 94-99, 1997.

(e) Neoplasms can be induced using techniques well known in the art, including but not limited to the use of chemical carcinogens, viruses, and radiation exposure. Alternatively, naturally occurring tumors can also serve as a source for cancerous cells. Tumor cells can then be cultured as described in Lehr *et al.*, *Anticancer Res.* 18(6A): 4483-4488, 1998.

The cell-based model can also be used to identify and/or characterize test compounds known to, or expected to, modulate the activity of P-gp. In examples of such methods, the cells are contacted (*e.g.*, concurrently or sequentially) with both the test compound and a compound known to be transported by P-gp (the effector molecule). The cells are then monitored to detect one or more effects caused by the effector compound, and the level of effect can be compared to animals that were

not treated with the test compound (or which received a different treatment regimen). Depending on the effector molecule used, different biological characteristics of the cells can be monitored to determine whether (and to what extent) the test compound influences P-gp interaction with the effector. Depending on whether the test molecule increases or decreases P-gp transport of the effector, the test molecule is identified as an agonist or antagonist of P-gp activity and can be selected for further characterization.

Compound transport across a membrane in cultured cells can be monitored using known techniques, for instance the radio-labeling method outlined in Schinkel *et al. J. Clin. Invest.* 96:1698-1705, 1995. Briefly, cells are grown in complete medium including L-glutamine, penicillin, streptomycin, and FCS and seeded on microporous polycarbonate membrane filters (3.0 μM pore size, 24.5 mm diameter, Transwell™ 3414, Costar Corp., Cambridge, MD) at a density of 2×10^6 cells per well. Medium at either the apical or basal side of the cell layer is replaced with complete medium containing the appropriate concentration of radiolabeled drug or drug candidate and cells are incubated at 37° C in 5% CO_2. Subsequently, 50 μl aliquots are taken from each compartment at various time intervals, for instance 1, 2, 3, and 4 hours. The appearance of radioactivity in the non-treated compartment is measured and presented as the fraction of total radioactivity added at the beginning of the experiment.

In some methods employing the provided cell systems, the level of radioactive effector compound transported into the cells is compared between cells that received treatment with a test compound (*e.g.*, a putative or potential P-gp activity agonist or antagonist) and those that did not receive such treatment, or between cells that received different test compound treatments (for instance, as to the test compound used, the method or timing of application, the amount applied, and so forth).

Methods of monitoring compound-uptake will in some instances be influenced by the compounds being studied.

This disclosure provides a mutation in the canine (particularly collie) *mdr1* gene, which results in a truncation of P-gp and leads to extreme sensitivity to ivermectin in animals homozygous for this mutant allele. The disclosure further provides methods and kits for screening for this mutation, in order to identify animals susceptible to toxicity from ivermectin and other neurotoxic compounds that interact with the P-gp transport protein. Also provided are whole-animal and cell-based model systems for studying compound interactions with P-gp using the *mdr1* truncation mutation described herein. It will be apparent that the precise details of the methods described may be varied or modified without departing from the spirit of the described invention. We claim all such modifications and variations that fall within the scope and spirit of the claims below.